AMPLIFLUOR™ DIRECT GENE SYSTEMS ASSAY PROTOCOL: ABI PRISM®

This protocol is adapted from Applied Biosystems protocol by James Cherry to meet the needs of 7900HT SDS.
This protocol is for use with Intergen's Amplifluor™ Cytokine Direct Gene Systems and Amplifluor™ Housekeeping Gene Systems. For additional technical inquiries, contact Technical Service at 800-465-8697 or tech.support@intergenco.com

BEFORE STARTING THE EXPERIMENT

Thermophilic Taq polymerase

The assay conditions described in this bulletin were developed with Platinum® *Taq* Polymerase (Life Technologies, Gaithersburg, MD). Certain "hotstart" thermophilic polymerases are not recommended with the described conditions. (Contact Technical Service for enzyme recommendations.)

PCR Reaction Vessel

Perform the assay with optically clear amplification tubes and caps or an optically clear PCR plate. The assay conditions described were developed using PE optically clear tubes (cat#N801-0933) and caps (cat#N801-0935).

Experimental Design

The required controls for each target of interest are the No Target Control and the Target Template Dilutions.

No Target Control (NTC)

This control is used to determine the limits of PCR sensitivity and tests reactions for possible background that may occur during amplification. The statistical significance of lowest detectable signal is determined by employing the threshold cycle (C_t) of NTC. This control also assesses the generation of primer-dimer PCR artifact or amplicon contamination of a kit reagent (see Troubleshooting).

Target Template Dilutions

Generation of a standard curve is required with each experiment and for each target of interest (i.e. one for each housekeeping target and one for each cytokine target). Serial 10 fold dilutions of the Target Template (10^7 to 10^1 copies per reaction) are amplified. Under the experimental conditions described in this bulletin, minimum sensitivity of 100 copies is expected. It is recommended that target dilutions be run in duplicate for increased accuracy.

Experimental Samples
Dilutions of experimental samples are generally not required. Run at least duplicates of all experimental samples.

REAL TIME ASSAY PROTOCOL

Step A. Thermocycling Conditions

1. Enter the parameters listed in Table 1 into the thermocyler.

Table 1: PCR Program

Stage	**1**	**2**		
	Denaturation	***Repeat: 40-45 cycles***		
		Melt	**Anneal/Extension**	
Step		**(1)**	**(2)**	**(3) Optional**
Temperature	**95°C**	**94°C**	**55°C**	**72°C**
Time (min.)	**5:00**	**00:15**	**1:00**	**0:40**

Note: *Data collection for the Amplifluor™ System should be performed at the annealing/extension step.*

2. Typically, ROX fluorescence is detected along with the signal of the fluorophores used to label the amplified product. Make sure that the ROX fluorescence detection is turned "OFF" when working with the Amplifluor™ systems.

Step B. Assay Procedure

1. Add 2 µl of H_20 to tubes designated as No Target Controls.

2. Using pipettes designated only for aliquoting DNA, prepare an initial working stock solution of 5 x 10^6 copies/µl by adding 5 µl of the Target Template (1 x 10^8 copies/µl) to 95 µl of H_20. Then, prepare 1:10 serial dilutions of the initial

stock (5 x 10^6 copies/μl) with TE to obtain Target Template concentrations of 5 x 10^5, 5 x 10^4, 5 x 10^3, 5 x 10^2, 5 x 10^1, and 5 x 10^0 copies/μl (Table 2).

Table 2: Preparing Target Template Dilutions

Final Concentration	Stock Solution	TE
1. 5 x 10^6 copies/μl	Working stock	
2. 5 x 10^5 copies/μl	5 μl of #1	45 μl
3. 5 x 10^4 copies/μl	5 μl of #2	45 μl
4. 5 x 10^3 copies/μl	5 μl of #3	45 μl
5. 5 x 10^2 copies/μl	5 μl of #4	45 μl
6. 5 x 10^1 copies/μl	5 μl of #5	45 μl
7. 5 x 10^0 copies/μl	5 μl of #6	45 μl

3. Aliquot 2 μl of the serially diluted control template (in duplicate, 5 x 10^0 to 5 x 10^6 copies/μl) and experimental samples (duplicate or triplicate).

4. Prepare the PCR " Master Mix" using the reagents (equilibrated at room temperature) listed in Table 3.

Table 3: Preparation of PCR "Master Mix"

Components of "Master Mix"	Volume per Reaction
DH_2O	**17.0 μl**
10X Reaction Mix A	**2.5 μl**
2.5 mm dNTP Mix	**2.0 μl**
20X Amplifluor™ Primer 1	**0.63 μl**
20X Primer 2	**0.63 μl**
"Hot Start" Polymerase (5 units/μl)	**0.2 μl (1 Unit)**
	23.0 μl

5. Place tubes in thermocycler and begin amplification.

EXAMPLE OF DATA ACQUISITION AND ANALYSIS

1. An assay using Amplifluor™ Hu GAPDH house keeping genes, was performed with serial dilutions of Hu GAPDH target DNA. All experimental procedures are as previously described. Real-time fluorescence was measured using the ABI PRISM® 7900.
2. A standard curve was generated by plotting the $\log_{10}$ [target copy #) of Hu GAPDH control template on X axis against the C_t value from serial dilutions of Hu GAPDH

target DNA on the Y axis (Figure 1). Standard curve, linear equation, and correlation coefficient (R^2) are automatically computed with the PRISM® software.

a. The standard curve is linear over 6 logs (10^1 to 10^6 copies) with correlation coefficient (R^2) of 0.998. Standard curve equation is Y= -3.647 X + 38.610

b. Relative copy # of unknown samples are determined according to standard curve equation (data not shown).

c. An amplification plot of the Control Template Dilutions (10^6 to 10^1 target copies per reaction) and No Target Control is presented in Figure 2. No signal is observed for the No Target Control; therefore, all C_t values for target dilutions are significant.

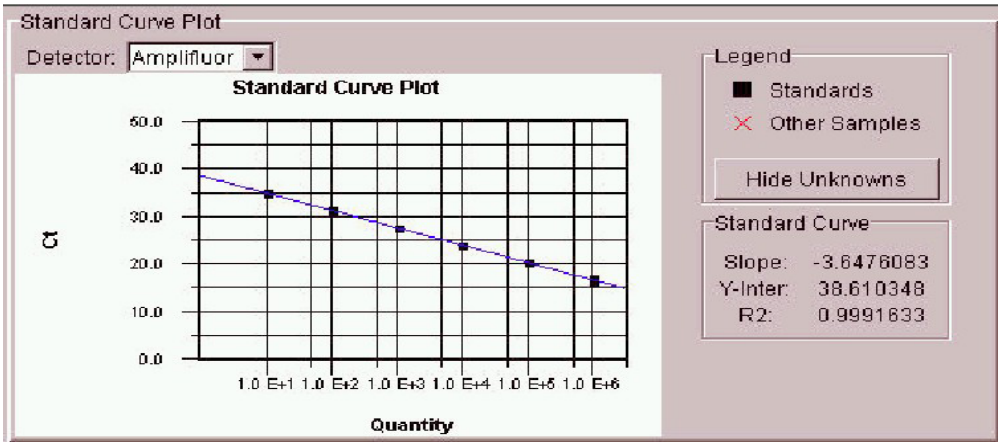

Figure 1 Standard Curve

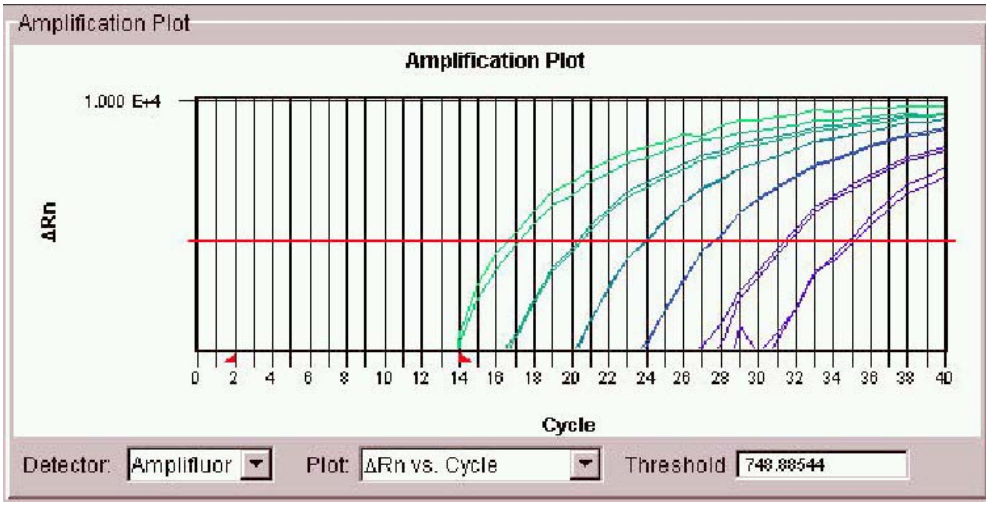

Figure 2 Amplification Plot

TROUBLESHOOTING

A. No amplification is observed.

Potential problem: PCR amplification is not initiated.

Recommendations:

1. Recheck the addition of kit reagents. Were the correct amounts of 10X Reaction Mix A or 2.5 mM dNTP Mix added? Was Taq polymerase included?
2. Recheck the thermocycler for proper temperature and time settings. Is the thermocycler cycling at 94°C/15 seconds and 55°C/1 min for 40 cycles? Is the initial 95°C/5 minutes denaturation step included?
3. Check the Taq polymerase to see if it is active.

B. No template Control shows amplification (positive C_t is obtained for NTC).

B1. Potential problems: Primer-dimer PCR artifacts.

As is anticipated with a PCR-based assay, some unavoidable PCR artifacts are expected even when the optimal assay conditions are employed.

Recommendations:

1. While primer-dimer reduces target sensitivity, C_t values for Target Template Dilutions and Experimental Samples that are 4 cycles lower than the No Target Control are statistically significant. A four cycle difference approximately equals a 10-fold difference in initial target concentration.
2. Use a "Hot Start" Taq polymerase in order to improve specificity. Platinum® *Taq* polymerase (Life Technologies) is recommended. "Hot start" enzymes available from other sources may require assay optimizations.
3. Do not amplify over 40-45 cycles.
4. Optimize the amplification conditions by increasing annealing/extension temperature to 58°C.

B2. Potential problems: PCR carry-over contamination.

Recommendations

1. Use fresh aliquots of assay reagents (10X Reaction Mix A, 2.5 mM dNTP Mix, Taq polymerase and DNase-RNase-free water).
2. Follow the recommendations described in the manual (Sec. 5.3. Laboratory Set-up and Precautions). PCR racks are the most likely source of PCR carry-over contamination. Decontaminate the racks as described in Sec. 5.3.